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TECHNOLOGY REPORT

A Cre Recombinase Transgene with Mosaic, Widespread Tamoxifen-Inducible Action

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Summary: Cre-mediated site-specific recombination allows conditional transgene expression or gene knockouts in mice. Inducible Cre recombination systems have been developed to bypass initial embryonic lethal phenotypes and provide access to later embryonic or adult phenotypes. We have produced Cre transgenic mice in which excision is tamoxifen inducible and occurs in a widespread mosaic pattern. We utilized our Cre excision reporter system combined with an embryonic stem (ES) cell screen to identify ES cell clones with undetectable background Cre activity in the absence of tamoxifen but efficient excision upon addition of tamoxifen. The CreER™ transgenic mouse lines derived from the ES cells were tested using the Z/AP and Z/EG Cre reporter lines. Reporter gene expression indicated Cre excision was maximal in midgestation embryos by 2 days after tamoxifen administration, with an overall efficiency of 5–10% of cells with Cre excision. At 3 days after tamoxifen treatment most reporter gene expression marked groups of cells, suggesting an expansion of cells with Cre excision, and the proportion of cells with Cre excision was maintained. In adults, Cre excision was also observed with varying efficiencies in all tissues after tamoxifen treatment. genesis 32:8–18, 2002.

INTRODUCTION

The use of transgenic and gene targeting technology has been a tremendous advantage for studying gene function in mice and making models of human disease. In many cases, however, transgene expression or gene inactivation results in embryonic lethality, so that only the earliest phenotype can be explored (Lobe and Nagy, 1998; Marth, 1996; Rajewsky *et al.*, 1996). For transgenic mice, an embryonic lethal phenotype may also preclude the establishment of mouse lines (Jhappan *et al.*, 1992; Lardelli *et al.*, 1996). A variety of strategies have been developed to bypass these problems (Lobe and Nagy, 1998). Of these, the most efficient ones use the Cre recombinase system (Nagy, 2000). Site-specific Cre recombinase is also the method of choice to achieve conditional gene knockouts (Takeda *et al.*, 1998; Tsien *et al.*, 1996).

The Cre recombinase enzyme of bacteriophage P1 catalyzes recombination between two 34 base pair *loxP* sites (Abremski *et al.*, 1983). If the *loxP* sites are placed in the same orientation on a strand of DNA, the outcome of this enzymatic reaction is the excision of the intervening DNA between the *loxP* sites (Abremski *et al.*, 1983; Hamilton and Abremski, 1984; Sauer, 1987; Sauer and Henderson, 1988). To achieve conditional transgene expression, a floxed (*loxP*-flanked) stop sequence is placed between a gene promoter and the transgene so that transgene expression does not proceed until Cre excises the stop sequence (Lakso *et al.*, 1992). For conditional gene knockouts, *loxP* sites are placed on either side of a critical exon, thus introduction of Cre recombinase leads to excision of the exon and effectively a gene knockout (St-Jacques and McMahon, 1996). These strategies allow the establishment of mouse lines with silent genetic alterations that can be activated by Cre-mediated excision. The Cre recombinase can be introduced, for example, by crossing the mouse line carrying the silent transgene/gene knockout with a Cre transgenic mouse. Depending on the expression of the Cre transgene, the genomic alterations can be made in a general or tissue-specific manner (Nagy, 2000). This approach allows establishment of transgenic lines that would otherwise be embryonic lethal, but introduction of Cre recombinase still does not allow temporal control apart from the onset of Cre expression. A refinement of the Cre/*loxP* approach has been the use of inducible Cre recombinases (Feil *et al.*, 1996; Hennighausen and Furth, 1999; Metzger *et al.*, 1995a; Utomo *et al.*, 1999). One way to render the Cre protein inducible is to fuse it to the ligand binding domain (LBD) of a hormone receptor so that the

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Cre/LBD fusion protein is retained in the cytoplasm until ligand addition allows nuclear localization and recombination at *loxP* DNA targets. Creating a ligand-inducible Cre fusion protein was first done using the LBD of the estrogen receptor (Metzger *et al.*, 1995b). A drawback of this approach was that endogenous ligand might cause background expression and treatment with estrogen would have biological consequences apart from activating the Cre protein. This problem was solved by developing modified LBD that preferentially recognize synthetic ligands (Brocard *et al.*, 1998; Feil *et al.*, 1996; Kellendonk *et al.*, 1996). The most successful of these has been the mutated estrogen receptor LBD that selectively binds tamoxifen (Brocard *et al.*, 1997; Danielian *et al.*, 1998; Feil *et al.*, 1996; Imai *et al.*, 2001a, 2001b; Indra *et al.*, 1999; Metzger and Chambon, 2001).

Several transgenic mouse lines with tissue-specific, tamoxifen-inducible Cre have been described (Danielian *et al.*, 1998; Imai *et al.*, 2001a, 2001b; Indra *et al.*, 1999; Kuhbandner *et al.*, 2000; Schwenk *et al.*, 1998). They allow conditional expression of a transgene in mice that have both the transgene and the Cre recombinase, after treatment with the tamoxifen ligand. However, we wished to create a mouse line with widespread, rather than tissue-specific, expression of an inducible Cre. Such a line would be useful to obtain mouse models for sporadic genetic disease and examine the consequences of transgene expression globally in an adult animal. The generation of transgenic lines with no background excision in the absence of ligand but a reasonable level of mosaic Cre excision upon addition of ligand appeared to be a challenging goal. To meet this goal, we used a screening approach in embryonic stem (ES) cells to identify lines with the desired window of expression, that is, no background excision but efficient induction upon ligand addition. We then generated mouse lines from the ES cell clones and measured their Cre activity using our reporter transgenic lines. This approach resulted in transgenic lines with no Cre activity in the absence of inducer, but widespread Cre excision after addition of tamoxifen. These mouse lines are valuable to create inducible genetic changes at defined timepoints of embryonic and adult life.

RESULTS

Selection for Transgene Expression in ES Cells

To achieve inducible recombinase activity, we used a fusion protein (CreER™) of Cre recombinase and the ligand binding domain of a mutant mouse estrogen receptor that does not bind the endogenous ligand 17β-estradiol at normal concentrations but retains affinity for the synthetic ligand 4-hydroxytamoxifen (Danielian *et al.*, 1998). The CreER™ fusion protein is sequestered in the cytoplasm until administration of tamoxifen, which allows nuclear localization of CreER™ and excision of *loxP*-flanked DNA targets. To obtain transgenic mice with widespread expression of CreER™, we used the strong pCAGGS promoter, consisting of the CMV early enhancer and chicken β-actin promoter (Niwa *et al.*, 1991) contained in the pCX expression vector. A PGK-puromycin cassette was inserted into the pCX-CreER™ vector to allow selection of ES clones with stable integration of the transgene.

We wished to obtain a level of expression that provided efficient excision in the presence of tamoxifen with the least background excision in the absence of tamoxifen. Because the level of expression of a given transgenic construct depends on the actual integration site, we decided to screen a large number of sites in ES cells for proper inducibility. We hypothesized that such an in vitro screen would enrich for integration events that function the same way when introduced in vivo. For our in vitro screen, we used ES cell lines that carried the Z/AP transgene (Fig. 1). Z/AP is a Cre reporter in which a *loxP*-flanked *lacZ*-neomycin resistance fusion gene (β-geo) is expressed prior to Cre excision, but after Cre excision the β-geo is excised and a second reporter, human placental alkaline phosphatase (hPLAP), is expressed instead (Lobe *et al.*, 1999). Thus, ES cells carrying the Z/AP transgene are G418 resistant because of β-geo expression after Cre excision. We electroporated the pCX-CreER™ transgene into Z/AP ES cells and selected for ES clones that were puromycin resistant, and thus carried the pCX-CreER™ transgene, and G418-resistant, indicating that Cre excision had not taken place in the absence of the tamoxifen ligand (Fig. 1b). These clones would therefore carry the pCX-CreER™ transgene but have no detectable background excision in the absence of tamoxifen. To then screen for ES clones that did have Cre excision activity in the presence of tamoxifen, the clones were grown in duplicate 96-well plates. One plate was grown in the absence of tamoxifen, the other was grown in the presence of tamoxifen (Fig. 1c). Both plates were double stained for *lacZ* and hPLAP expression to visualize Cre activity. We selected clones that displayed *lacZ* expression but no hPLAP activity in the absence of tamoxifen but intensive hPLAP staining in the presence of tamoxifen, thus indicating tamoxifen-dependent Cre excision (Fig. 1d). From this screen, 9 out of 288 ES cell clones were identified fulfilling this double criteria.

Two of the ES cell lines identified in this way, designated BD8 and CA6, were used for embryo aggregation to generate chimeric mice, which were bred to CD1 females to identify germline transmitting chimeras and obtain transgenic offspring. To avoid background reporter gene expression carried over from the ES cells, the pups were genotyped to identify those that carried the CreER™ transgene but not the Z/AP transgene that was present in the ES cells. These two CreER™ mouse lines have been maintained in a 129SvEV inbred background, but the expression analysis described below was done in 129SvEV × CD1 mice. The two CreER™ lines gave the same Cre excision activity and the data shown is representative of both lines.

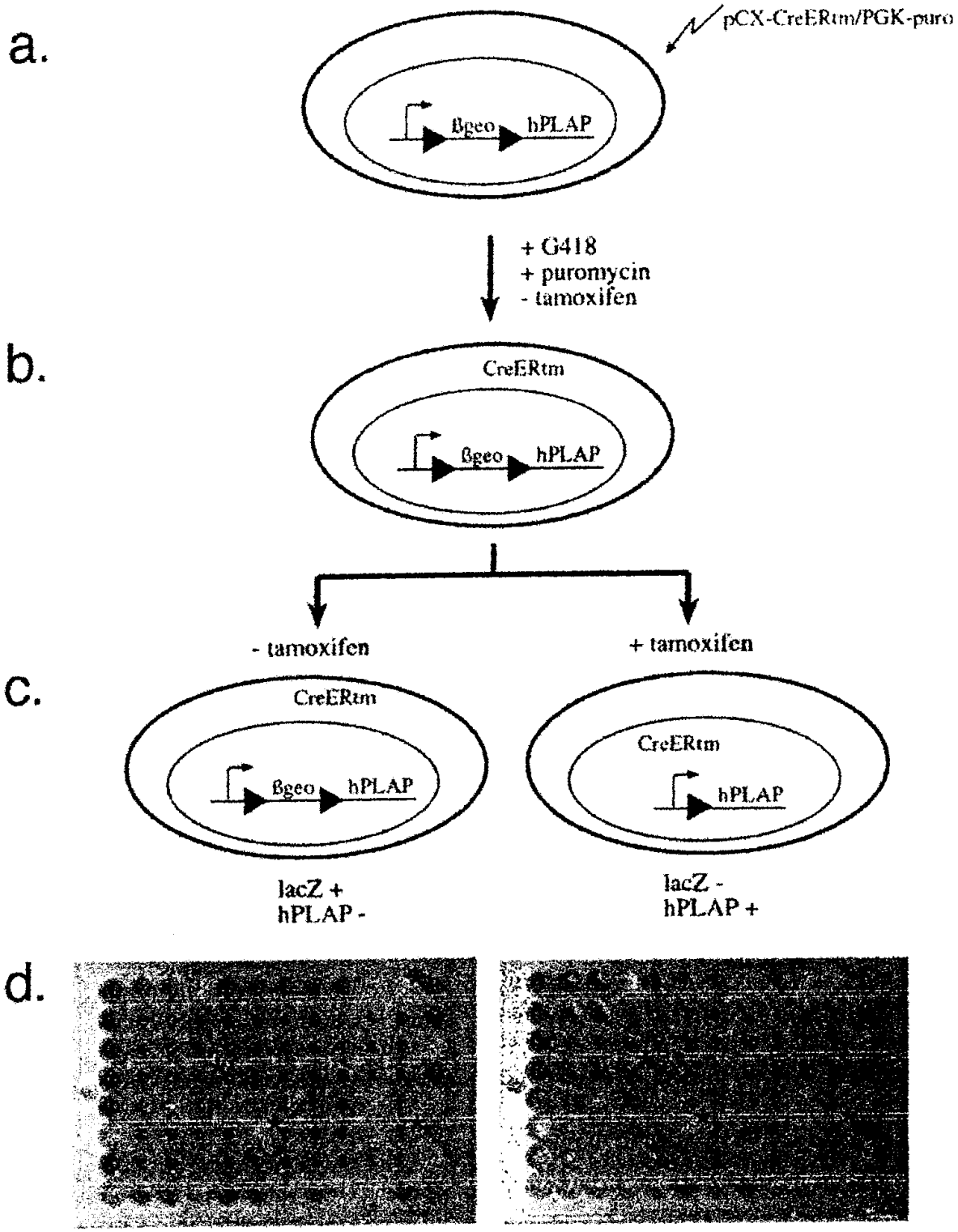

FIG. 1. Screen for ES cell clones expressing CreER™. (a) The pCX-CreER™ vector carrying a puromycin-resistance gene was introduced into Z/AP ES cells. (b) Cells were grown without tamoxifen to maintain CreER™ in the cytoplasm and in the presence of puromycin and G418 to select for the CreER™ plasmid and lack of recombination of the Z/AP transgene, respectively. (c) Cells were then plated in duplicate and grown in the absence or presence of tamoxifen. (d) Plates were double stained to identify ES clones that were *lacZ*-positive (blue stain) in the absence of tamoxifen and hPLAP-positive (purple stain) in the presence of tamoxifen, thus indicating a lack of background activity and efficient Cre excision with ligand addition. Some clones with the correct pattern are indicated with asterisks.

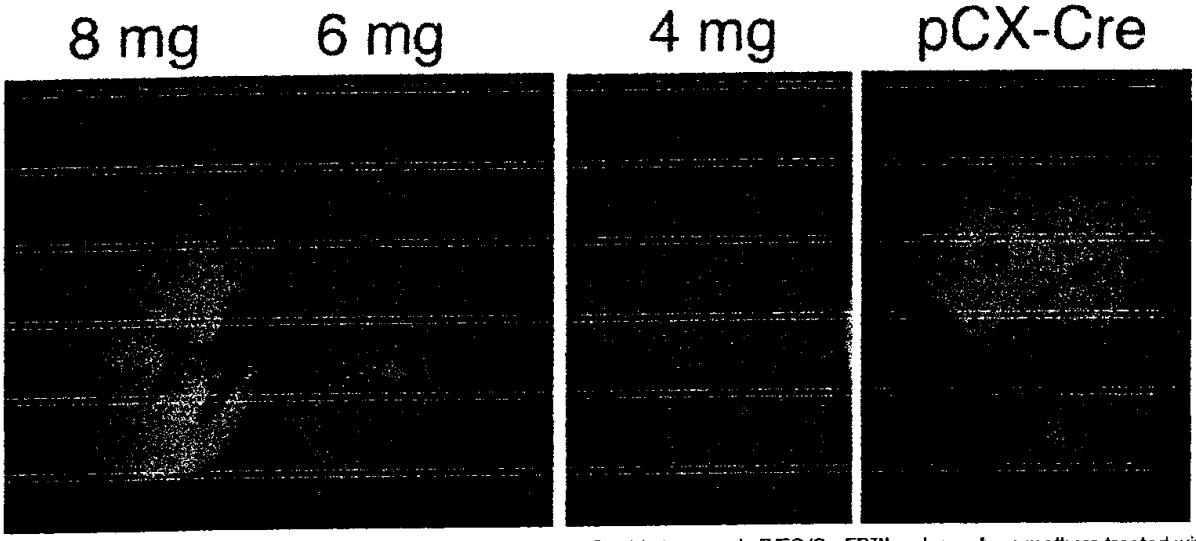

FIG. 2. Tamoxifen-induced Cre excision in Z/EG/CreER™ embryos. Double transgenic Z/EG/CreER™ embryos from mothers treated with 4, 6, and 8 mg of tamoxifen are shown. With 4 mg of tamoxifen some GFP expression is present in the forelimb, whereas with 6 and 8 mg of tamoxifen progressively more GFP fluorescence is present. For comparison, a double transgenic Z/EG/pCX-Cre embryo with Cre activity in all cells is shown on the right.

Tamoxifen-Dependent Cre Excision in Embryos

We tested the activity of the pCX-CreER™ transgene by crossing the mice to our Z/AP and Z/EG Cre excision reporter mice. As described above, the Z/AP reporter provides *lacZ* expression before Cre excision and hPLAP reporter gene expression after Cre excision (Lobe *et al.*, 1999). The Z/EG reporter similarly provides *lacZ* expression before Cre excision but after Cre excision and removal of the *lacZ* reporter, an enhanced green fluorescent protein (EGFP) reporter gene is expressed (Novak *et al.*, 2000).

We first used the Z/EG reporter mice to determine the optimal tamoxifen dose and the time required for Cre excision and consequent reporter gene expression. Single transgenic pCX-CreER™ males were crossed to Z/EG females. The pCX-CreER™ transgene was carried in the paternal genome to avoid background Cre excision from the maternal genome (Lewandoski *et al.*, 1997). The pregnant Z/EG females were treated with 4–10 mg tamoxifen at 9.5 dpc and embryos were dissected at 12.5 dpc. Double transgenic CreER™/Z/EG embryos could be easily identified by their green fluorescence (Fig. 2). With 4 mg tamoxifen, almost no GFP expression was observed in double transgenic embryos, whereas with 6 mg tamoxifen more widespread GFP expression was seen. The 8 and 10 mg doses of tamoxifen provided equal and maximal efficiency of Cre excision, as measured by the number of GFP-positive embryo cells. The embryos also developed normally. However, the 10 mg of tamoxifen was toxic for approximately 1 in 3 females, whereas with 8 mg of tamoxifen, only 1 in 8 females were affected. We therefore used 8 mg of tamoxifen in further studies. The fraction of GFP-positive cells was quantitated by FACS and by counting cells under a microscope. Both analyses indicated a range of 5–6% of the cells were GFP-positive (data not shown). As a control, double transgenic pCX-NLS-Cre/Z/EG embryos, which should have Cre excision in all cells, had 60–90% GFP-positive cells (Fig. 2).

To determine the time course of Cre excision, tamoxifen was administered at 9.5 dpc, embryos were dissected at 9.5, 10.5, 11.5, and 12.5 dpc, and the number of GFP-positive cells was determined by FACS. No GFP expression was observed at 9.5 or 10.5 dpc, whereas 11.5 and 12.5 dpc embryos appeared to have a similar level of GFP. FACS analysis indicated that 3 to 7% of cells in both the 11.5 and 12.5 dpc embryos were GFP positive (three embryos per group). Therefore, in the double transgenic CreER™/Z/EG embryos, there is a lag of approximately 2 days between tamoxifen administration and reporter gene expression at a detectable level. This delay in reporter gene detection is probably due to the time required for excision by Cre, transcription of the reporter transgene, and translation of the reporter protein.

Measurement of Cre Excision at the Cellular Level Using Z/AP Mice

Cre excision has been shown to vary in efficiency of excision between different *loxP* targets (Hebert and McConnell, 2000). This variability may be a result of different accessibility of the target *loxP* sites that lie in different regions of the genome. To measure the activity of our CreER™ lines on a different target, we used our Z/AP reporter mice. In addition, the Z/AP mice incorpo-

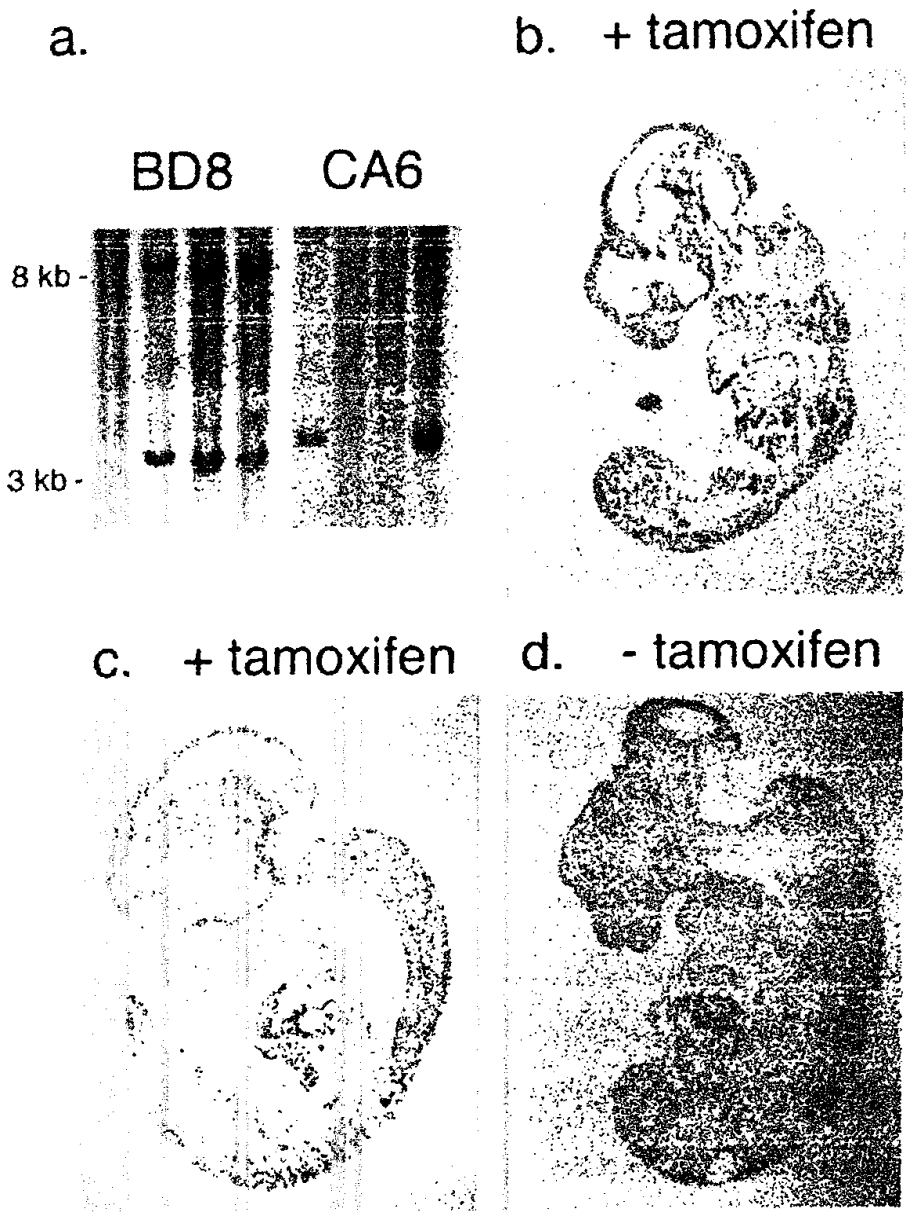

FIG. 3. Tamoxifen-induced Cre excision in Z/AP/CreER™ embryos. Z/AP females were crossed with CreER™ males and given 8 mg of tamoxifen at 9.5 dpc. (a) Embryos were dissected at 11.5 or 12.5 dpc and genotyped using the yolk sac for Southern blot. BD8 has two copies of the CreER™ transgene and CA6 has a single copy. Lanes 2, 3, and 4 for BD8 and 1 and 4 for CA6 are positive for the CreER™ transgene. (b–d) Sections of Z/AP/CreER™ 11.5 dpc (b) or 12.5 dpc (c, d) embryos stained for hPLAP activity. Embryos in (b) and (c) are from mothers treated with tamoxifen; the embryo in (d) is from an untreated mother. Without tamoxifen, no hPLAP staining is seen, whereas with tamoxifen, sporadic widespread hPLAP staining is apparent.

rate a second reporter (hPLAP) that is more easily visualized at the cellular level than GFP fluorescence

Single transgenic pCX-CreER™ males were crossed to Z/AP female mice At 9 5 dpc, the pregnant females were injected intraperitoneally with 8 mg of tamoxifen The embryos were then dissected at 11 5 or12.5 dpc and stained for *lacZ* and hPLAP activity to reveal sites of Cre excision (Fig 3). Each embryo was also genotyped for the CreER™ transgene using DNA extracted from the yolk sac (Fig 3a). Double transgenic CreER™/Z/AP embryos were positive for hPLAP activity throughout all tissues in both 11 5 and 12.5 dpc embryos (Fig. 3b, c). In 12 5 dpc but not 11 5 dpc embryos, much of the hPLAP-positive cells occurred in patches, suggesting most Cre excision occurred by 11.5 dpc and cells with Cre excision had expanded by 12.5 dpc (Fig. 4).

Some tissues, such as muscle, heart, liver, blood vessels, and CNS showed a high proportion of cells with hPLAP stain This could be a result of higher expression levels of CreER™ and Z/AP in those tissues, such as in muscle where the pCAGGS promoter is highly active (Lobe *et al*., 1999; Novak *et al*., 2000). In addition,

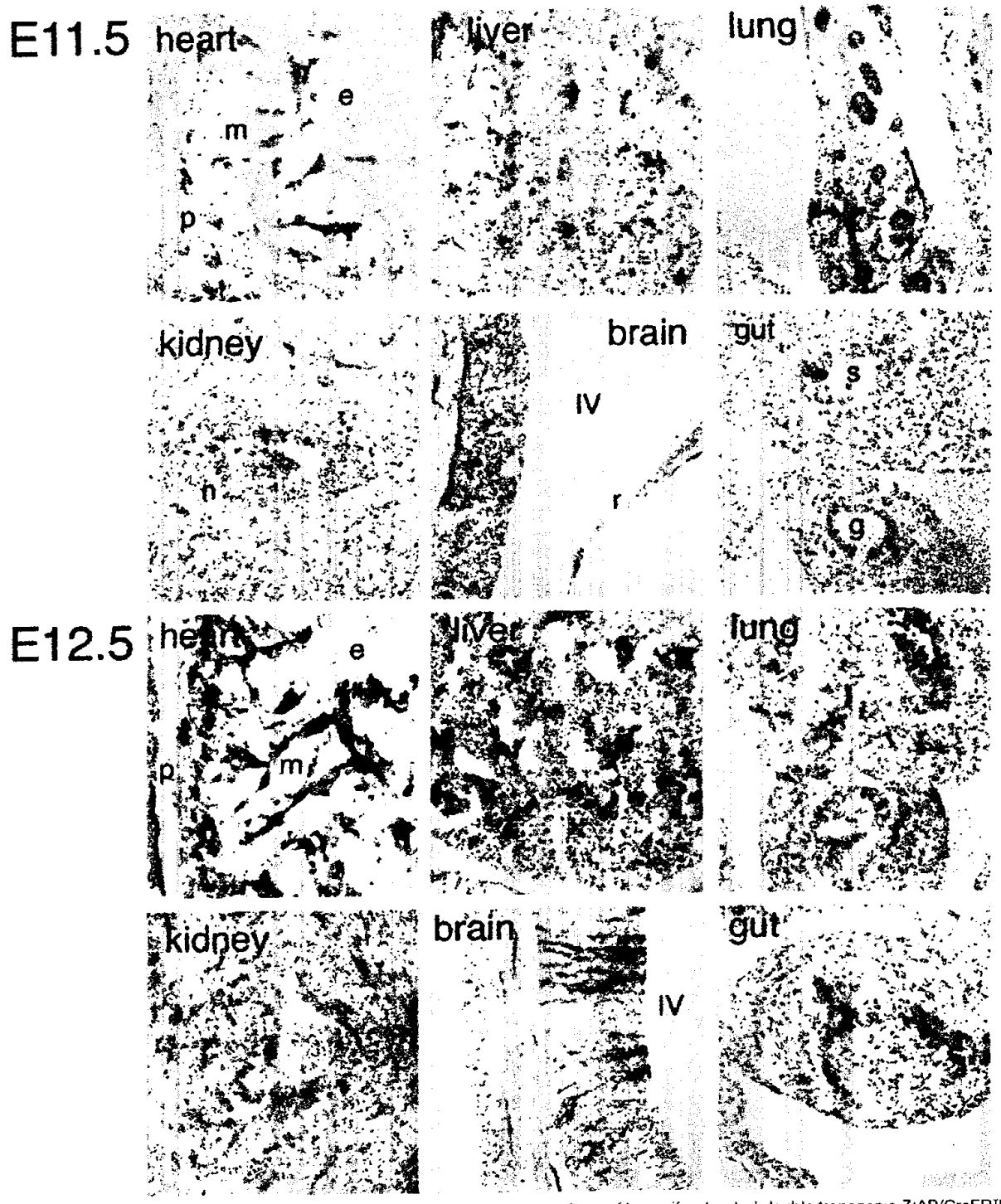

FIG. 4. Cre excision in organs of Z/AP/CreER™ embryos. High magnification views of tamoxifen-treated double transgenic Z/AP/CreER™ embryos at 11.5 dpc (upper rows) reveals cells in all organs with Cre excision, indicated by the purple hPLAP stain. At 12.5 dpc (lower rows), hPLAP staining of the same organs marks mostly groups of cells with Cre excision. e, endocardium; m, myocardium; p, pericardium; n, mesonephric tubule; IV, fourth ventricle; r, roof of fourth ventricle, s, stomach; g, midgut.

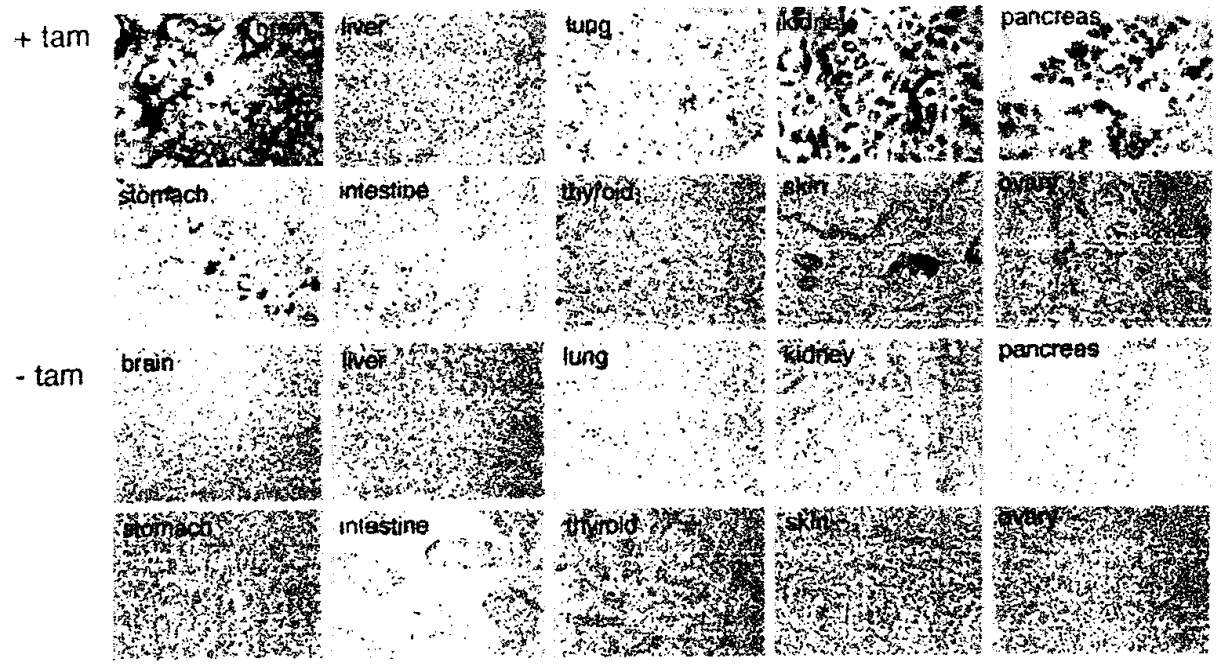

FIG. 5. Widespread sporadic Cre excision in tamoxifen-treated Z/AP/CreER™ adult mice. Tissue sections from 5-week-old double transgenic Z/AP/CreER™ mice were stained for nPLAP activity to identify sites of Cre excision. The top two rows are from tamoxifen-treated mice and the bottom two rows are from untreated mice. Treated animals have Cre excision to a varying extent in all organs, whereas untreated animals have no detectable Cre excision.

greater access to tamoxifen may account for the strong signal in the blood vessels and liver.

Single transgenic (Z/AP or CreER™) and nontransgenic littermates showed no hPLAP activity. More importantly, double transgenic CreER™/Z/AP embryos from females that were not treated with tamoxifen were also completely negative for hPLAP stain, indicating that without addition of the tamoxifen ligand, the CreER™ does not recombine *loxP* targets (Fig. 3d).

Tamoxifen Induction of Cre Excision in Adult Animals

The efficiency of Cre excision in adult CreER™ transgenic animals was also measured using the Z/AP and Z/EG reporter mice. CreER™ males were crossed with Z/AP females and double transgenic CreER™/Z/AP animals were treated with 8 mg of tamoxifen when 4 weeks old. As a negative control, half of the double transgenic animals were not given tamoxifen. Tissue samples were taken 5 days after treatment and stained for hPLAP activity. All samples from tamoxifen-treated animals showed some Cre excision in an apparently sporadic pattern, indicated by hPLAP staining (Fig. 5). Tissues varied in the number of cells that had undergone Cre excision. Muscle and pancreas in particular had a high proportion of hPLAP-positive cells. The nontamoxifen treated double transgenic animals had no cells with hPLAP staining. Because the stain for hPLAP activity is a sensitive enzymatic assay, this indicates that there is no background Cre excision in animals up to 5 weeks old.

We also examined the levels of Cre excision in 4- to 5-week-old adult mice using the Z/EG reporter line. When tamoxifen was administered to double transgenic CreER™/Z/EG animals, green fluorescence was observed in the ears and paws, where the hair does not cover the skin, after 7 to 8 days (Fig. 6a). Dissection of the mice revealed that the organs had undergone sporadic Cre excision, revealed by areas of green fluorescence (Fig. 6). Cre excision occurred at the highest levels in muscle, pancreas, and blood vessels, but some Cre excision was apparent in all tissues, similar to the pattern of Cre excision seen in the CreER™/Z/AP double transgenic mice.

A few double transgenic mice were also treated topically with tamoxifen by application to the ear. This resulted in green fluorescence at 7 to 8 days after treatment, indicating Cre excision, although we noticed that excision was not restricted to the site of application. Rather, mosaic areas of green fluorescence were observed in both ears and all paws, suggesting the tamoxifen/DMSO solution is readily absorbed in the bloodstream (data not shown).

We found that some older double transgenic CreER™/Z/AP and CreER™/Z/EG mice had background Cre excision in the absence of tamoxifen. For the Z/AP reporter, background hPLAP expression was observed in

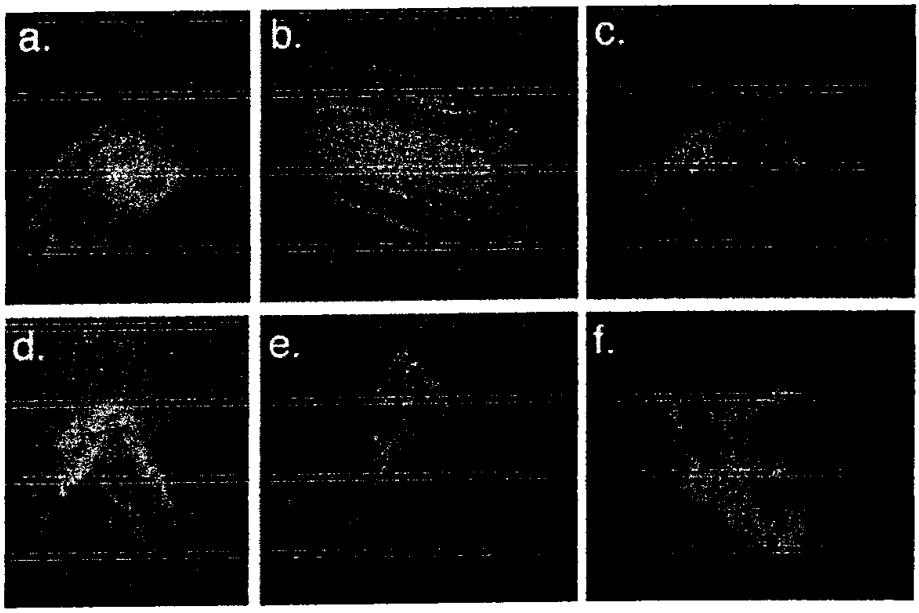

FIG. 6. Cre activity in Z/EG/CreER™ mice. Z/EG/CreER™ mice were treated with tamoxifen and Cre excision was visualized by GFP fluorescence. (a) Left foreleg; (b) Skeletal muscle; (c) Heart; (d) Pulmonary artery; (e) Intestine and villi; (f) Pancreas.

cardiac muscle after 6 weeks of age, whereas for the Z/EG reporter, green fluorescence appeared in the muscle at 13–15 weeks of age. This background activity is probably caused by the high expression of CreER™ in muscle by the pCAGGS promoter.

DISCUSSION

To achieve inducible genetic changes in transgenic mice, we developed CreER™ transgenic mice with tamoxifen-inducible Cre recombinase and measured the excision activity in Cre reporter mouse lines. Our strategy of using ES cells to screen for CreER™ lines with the correct level of transgene activity represents an efficient way to identify transgenic clones with a required characteristic or expression profile. Both of the CreER™ ES clones that were selected by their inducible Cre excision activity in vitro provided mouse lines with widespread and efficient Cre excision in the presence of tamoxifen and virtually no background activity in the absence of tamoxifen in vivo. A similar approach can be taken for tissue-specific expression by introducing the transgene of interest and differentiating the ES cell clones to test for correct tissue-specific activity (Ding *et al.*, 2001).

Cre recombinase can vary in efficiency between *loxP* sites at different integration sites, therefore measuring Cre activity in one reporter line might not reflect activity for other *loxP* targets (Hebert and McConnell, 2000). We used two different Cre reporter lines, Z/AP and Z/EG, that represent *loxP* targets at two different integration sites. They also provide two different readouts, an enzymatic assay with hPLAP and fluorescence with GFP. By using different Cre reporter lines, we could measure the scope of inducible Cre activity and possibility of background Cre excision for two different transgene integration sites. For both reporters, tamoxifen-induced excision had a 5–10% excision rate compared to a general Cre deletor line. Although excision was observed in all tissues, both Cre reporter lines exhibited higher Cre activity in muscle, pancreas, nervous system, and blood vessels. This probably reflects the strong activity of the pCAGGS promoter driving Cre expression in those tissues and possibly the access of tamoxifen, which is highly water-insoluble. Cre excision in the absence of tamoxifen was observed in cardiac muscle of some CreER™/Z/AP animals after 6 weeks of age and in muscle of CreER™/Z/EG animals after 13 weeks of age. The difference in onset of this background activity likely reflects the greater sensitivity of the hPLAP enzymatic reporter, although the Z/AP transgene may also be more accessible to Cre excision (Hebert and McConnell, 2000).

The Z/AP and Z/EG reporter lines also provided complementary information about the pattern of Cre excision. The enzymatic hPLAP reporter showed in tissue sections that Cre excision occurred in scattered cells throughout the embryo 2 days after tamoxifen administration, but 1 day later was represented mainly in patches of cells. The GFP reporter allowed quantification of the number of cells, showing that the proportion of cells with Cre excision remained similar between 2 and 3 days after tamoxifen treatment. Together this indicates that most tamoxifen-induced Cre excision has taken place by 2 days, and subsequent cell division provides expansion of cells with Cre excision. This dynamic may be used to trace cell lineage or follow expansion from a single stem or progenitor cell. In adults the hPLAP reporter showed isolated cells with Cre excision even 5

days after tamoxifen treatment, marking excision in cells that were not proliferately active.

The combination of Cre recombinase and an inducible system to activate transgene expression has some unique features. In classical inducible transgene systems, the inducing agent must be continuously present to maintain transgene expression. This can be useful to look at the consequences of a pulse of gene expression or to test if a phenotype produced by a transgene is reversible (Felsher and Bishop, 1999). However, in many cases the aim is instead to study the effect of turning on a transgene in a cell and all its descendents, requiring long-term application of the inducer. The continuous presence of the inducing agent can itself have an effect on the phenotype or affect the health of the animal. For this purpose an inducible Cre line is more suitable because, unlike other inducible expression systems, a pulse of Cre activity makes a permanent genomic alteration in a cell and that change is inherited by all the cell's progeny. Cre can be rendered inducible at the transcriptional level by using an inducible promoter to regulate its expression (Blau and Rossi, 1999; Furth, 1997; Gossen and Bujard, 1992; Kuhn *et al*., 1995), or at the protein level by fusing it to a hormone ligand binding domain to retain it in the cytoplasm until ligand addition (Brocard *et al.*, 1998; Feil *et al.*, 1997; Kellendonk *et al.*, 1996 , 1999; Metzger *et al.*, 1995b; Shockett and Schatz, 1997; Verrou *et al.*, 1999). The most successful has been the CreER™ used in this study (Feil *et al.*, 1997). A mouse line with general expression of tamoxifen-inducible Cre recombinase was recently reported, but excision activity was analyzed at the cell level only for skin, where Cre induction was most efficient (Metzger and Chambon, 2001).

Reports on using tissue-specific CreER™ and our results here indicate that CreER™ provides inducible excision in approximately 5–10% of cells. The recent development of Cre fusion proteins with two or three ligand binding domains may increase the efficiency of Cre excision for the tamoxifen-inducible approach and/or decrease the required dose of tamoxifen (Indra *et al.*, 1999). In our experiments, 8 mg of tamoxifen was the optimal dose but was occasionally toxic. At the levels of tamoxifen we are using, the toxicity seems to be a result of inflammation caused by the carrier (corn oil) in the peritonium rather than the drug. Although the use of 4-hydroxytamoxifen has been recommended because it is more active in vivo than tamoxifen, we observed it was more toxic than tamoxifen even though a lower concentration was required.

Transgenic mouse lines with widespread expression of an inducible Cre recombinase allow the exploration of late embryonic and adult gene functions. In many instances it is desirable to make genetic changes to a few cells in an inducible manner. Such instances include creating mouse models for human sporadic genetic disease, knocking out or activating gene expression in a limited number of cells and following their fate, or determining whether a phenotype is cell autonomous. In such studies, the CreER™ mice described here, with a widespread inducible Cre recombinase that makes permanent genomic alterations to cells and their progeny, is ideal.

METHODS

Transgene Construct

pBSCreER™ was a gift from Dr. Andrew McMahon (Harvard) (Danielian *et al.*, 1998). The 2 kb ApaI/EcoRI fragment of CreER™ was blunted and subcloned into the filled-in EcoRI site of the pCX expression vector (Niwa *et al.*, 1991). The PGK-Puromycin cassette (1.7 kb) was excised with NotI and SfiII from pPGKPuro (a gift of Dr. Kerry Tucker [Tucker *et al.*, 1996]) and cloned into the end-filled Hind III site in pCX-CreER™.

Electroporation and ES Cell Selection

The vector backbone of pCXCreER™-PGKPuro was excised with SfiI/ScaI. The fragment containing CreER™-PGKPuro was introduced by electroporation into the R1 ES cell line (Nagy *et al.*, 1993) containing a single copy of the Z/AP transgene (BE3) (Lobe *et al.*, 1999). Selection was carried out in ES cell media containing 150 μg/ml G418 (GIBCO-BRL 11811-031) and 1.5 μg/ml puromycin (Clontech 8052-1). The 288 colonies resistant to neomycin and puromycin were picked into 96-well plates. Each plate was split to three duplicate plates; one plate was frozen as stock and the other two were used for the tamoxifen induction experiment.

4-hydroxytamoxifen (Sigma H-7904) was dissolved in ethanol at a concentration of 2 mM as a stock solution. ES cell media containing 200 nM of 4-hydroxytamoxifen was prepared fresh each day before addition to one of the duplicate plates. The control plates were fed with ES cell media without tamoxifen. Three days after addition of tamoxifen, the cells were washed with PBS, fixed with 0.2% glutaraldehyde for 5 min, washed three times with PBS, and stained with *lacZ* solution for 4 h at 37°C, as described (Lobe *et al.*, 1999). The cells were then washed with PBS and incubated in prewarmed PBS at 75°C for 30 min to inactivate endogenous alkaline phosphatase. hPLAP staining was completed in AP staining solution for 30 min as described (Lobe *et al.*, 1999).

Colonies with more than 90% of cells staining blue for *lacZ* expression before tamoxifen and more than 98% staining purple for hPLAP expression after tamoxifen treatment were identified as clones with no leakage for Cre activity and efficient Cre excision with tamoxifen. We also tested different tamoxifen concentrations and the level of Cre activity as measured by hPLAP-positive cells. No difference was found at a range of 100–500 nM of tamoxifen. Nine out of 288 colonies met the criteria and were expanded.

Generation of Transgenic Mice

Three of the ES cell lines were aggregated with eight-cell embryos (Wood *et al.*, 1993). Germline transmission chimeras were identified for two lines (BD8 and CA6). Southern blot showed the BD8 line carries two copies of

the CreER™ transgene, whereas the CA6 line contains a single copy of the transgene.

The CreER™ and Z/AP transgenes were segregated in progeny of the BD8 and CA6 CreER™ chimeric males by genotyping for CreER™ by Southern analysis (Danielian *et al.*, 1998) and for Z/AP by *lacZ* staining the ear biopsy (Lobe *et al.*, 1999). Male mice that tested positive for the CreER™ transgene and negative for *lacZ* staining were selected to cross with female Z/EG or Z/AP mice.

Cre Excision Induced by Tamoxifen

Tamoxifen (Sigma T-5648) was dissolved in corn oil (Sigma C-8267) at a concentration of 20 mg/ml by rotating at room temperature for 6 h. The solution was kept protected from light at 4°C for no more than 3 weeks. For induction of Cre excision in embryos, 4–10 mg of tamoxifen was administered by intraperitoneal injection of the mother at 9.5 days postcoitum (dpc). Embryos were harvested at 9.5, 10.5, 11.5, and 12.5 dpc. For induction in adults, 6–10 mg of tamoxifen was injected and tissues were analyzed 5 days after tamoxifen treatment. Untreated control animals were given corn oil without tamoxifen.

Genotyping

DNA was extracted from tail and yolk sac samples using the salt-chloroform method (Mullenbach *et al.*, 1989). DNA samples were digested with EcoRV. The trangene was detected by Southern analysis using an EcoRI/XhoI fragment of the 3′ end of the Cre coding sequence as a probe (Danielian *et al.*, 1998)

Flow Cytometry

Embryos were minced with a scalpel and digested with 1 mg/ml Collagenase type III (GIBCO-BRL 17102-013) at 37°C for 30–60 min, according to the size of the embryos. The cell suspension was spun briefly and the cell pellets were resuspended in PBS and passed through a 30 μM nitex nylon mesh (B/SH Thompson, cat. HCS-30). The cells were analyzed on a Becton Dickinson FACScan Flow Cytometer, using a 480 nm laser for excitation and 530 nm emission for GFP (Novak *et al.*, 2000).

Whole-Mount and Sectioned Tissue Staining

Tissue samples were dissected, fixed, sectioned, and stained as described (Lobe *et al.*, 1999; Novak *et al.*, 2000). Whole-mount samples were photographed on a Leica M3 dissecting microscope using a CoolSnap digital camera and software package. Tissue sections were photographed on the same microscope or a Zeiss Axiophot compound microscope, also using the CoolSnap digital camera and software package.

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